Age-related loss of naïve T cells and dysregulation of T-cell/B-cell interactions in human lymph nodes

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Summary

In this study we analysed the effects of age on T and B lymphocytes in human lymph nodes by comparing lymphocyte subsets in paraffin sections from lymph node tissue taken from healthy young and elderly people. We demonstrate that the relative number of $CD8^+$ T cells decreases with age but that the relative number of $CD4^+$ T cells does not. There is also a very pronounced age-dependent loss of $CD45RA^+$ naïve T cells. The number and size of follicles and the relative number of $CD20^+$ B cells are similar in young and elderly donors. For polymerase chain reaction analysis of the T-cell receptor (TCR) repertoire the TCR-γ gene rearrangements were used as a marker of clonality. This is a reliable tool to detect not only clonal TCR-$\gamma\delta$ populations but also TCR-$\alpha\beta$ populations. Young donors with clonal T-cell expansions in their lymph node tissue do, however, have a higher number of $CD20^+$ B cells, a higher relative size of germinal centres compared to the follicle mantles and a higher number of immunoglobulin M-expressing cells than young donors without evidence of clonal T-cell expansions. Corresponding changes are not observed in elderly donors with clonal T-cell expansions in their lymph node tissue. In summary our findings demonstrate characteristic effects of aging on human lymph node tissue, the most striking feature being the depletion of naïve T cells and the apparent dysregulation of T-cell/B-cell interactions in old age.

Keywords: aging; B lymphocytes; human lymph nodes; T lymphocytes

Introduction

Infectious diseases occur more frequently and have a more severe clinical course in the elderly[1] and the efficacy of vaccination is low.[2] This is because of age-related changes within the immune system. The cumulative effects of aging on the haematopoietic stem cell and lymphoid progenitor pools in the bone marrow and the thymus play an important role in the diminution of immune responsiveness in the elderly.[3] Typical age-related changes in lymphocyte subpopulations are also believed to have functional consequences.[4,5] Thus, aging is associated with an increase in the number of antigen-experienced cells and a decrease in the number of naïve T cells in the peripheral blood[6] as well as an increase in the number of natural killer and natural killer/T cells.[7] Antigen-experienced T cells may occur as large expanded clones which dominate the repertoire, have lost the important costimulatory molecule CD28 and have a polarized type 1 cytokine production.[8] These clonal T-cell accumulations have been shown to be associated with lack of antibody production following immunization.[8] Changes in the B-cell compartment could also account for the declining immunological responsiveness in old age.[9] Changes occur in the B-cell repertoire similar to those observed in the T-cell pool.[9–12] Aging also affects the quality and quantity of the humoral immune response with decreased serum immunoglobulin concentrations and a low number of antigen-specific, immunoglobulin-secreting B cells. In addition, changes in antibody specificity, isotype and affinity have been described as typical features of old age.[11] Antibodies produced in aged mice are known to have lower affinity and to be less protective compared to antibodies from young animals.[13] This is not surprising as aging negatively affects the germinal centre

reaction in secondary lymphoid tissues of mice[14] and leads to a reduced formation of germinal centres in response to stimulation with tetanus toxoid.[15]

As an integral component of the immune system, lymph nodes play a crucial role in facilitating the processing of antigens and their presentation to T and B lymphocytes. Lymph nodes are the termination point of afferent lymphatic vessels that drain lymph from most body compartments, collect antigens and dendritic cells from peripheral tissues, recruit naïve lymphocytes from the blood and provide the microenvironment for primary and secondary immune responses.[16] While most studies on the effect of aging have so far focused on lymphocytes from the peripheral blood, potential age-related changes in the T-cell and B-cell areas of human lymph nodes are still insufficiently characterized. It was therefore the aim of this study to investigate the effects of aging on the microarchitecture of human lymph nodes from the young and from elderly people.

Materials and methods

Tissue

Formalin-fixed, paraffin-embedded inguinal, cervical, or axillar lymph node tissue obtained from 38 donors was collected from the archives of the Institute of Pathology at the Medical University of Innsbruck: 26 from elderly individuals (mean age 75 years, range 67–88) who underwent pelvic ($n = 20$) or cervical ($n = 6$) vascular reconstruction and 12 from young patients (mean age 11 years, range 1–20) in whom lymphadenectomy was performed for routine diagnosis of cervical or axillar lymphadenopathy. The characteristics of the donors are shown in Table 1. Cases with lymphoid and non-lymphoid neoplasms, Castleman's, Kikuchi's and Rosai–Dorfman's diseases, pronounced sinus histiocytosis, lymph node necrosis, progressive transformation of germinal centres, folliculolysis, dermatopathic lymphadenopathy, eosinophilia, or infectious mononucleosis, as well as those with granulomatous reactions and suppuration, were excluded. The study was approved by the ethical committee of the hospital.

Immunohistochemistry and immunofluorescence

Immunohistochemistry was performed using an automated immunostainer (Nexes, Ventana, Tucson, AZ); the streptavidin–biotin peroxidase technique with diaminobenzidine as chromogene was applied. The primary antibodies were diluted in a 1% solution of bovine serum albumin in phosphate-buffered saline (pH 7·4) and incubated for 30 min at room temperature. All slides were stained with monoclonal antibodies against CD4, CD8, CD45RA, CD20, immunoglobulin G (IgG) and IgM. All antibodies, their dilutions and pretreatment conditions are listed in Table 2.

Manual double stains for CD20 and IgD were performed to distinguish between the mantle zone and the germinal centre. Anti-CD20 antibodies stain all the B cells organized in the follicles whereas anti-IgD antibodies stained solely the mantle cells. Paraffin-embedded tissue sections were deparaffinized in xylene, followed by hydration in ethanol. The slides were cooked in citrate buffer (pH 6·0) in a microwave oven for epitope retrieval as listed in Table 2. The slides were removed from the oven and allowed to cool for 30 min at room temperature. After cooling, sections were incubated for 20 min with standard blocking reagent (Roche, Mannheim, Germany). Monoclonal mouse anti-human CD20 (NeoMarkers, Fremont, CA) and anti-IgD rabbit polyclonal (Dako, Glostrup, Denmark) antibodies were incubated for 60 min. The slides were then rinsed in Tris buffered saline (TBS) buffer containing 0·1% Tween-20 (Merck, Hohenbrum, Germany). Horseradish peroxidase-conjugated goat anti-mouse (Dako) and biotinylated swine anti-rabbit (Dako) antibodies were used for detection. After incubation for 30 min at room temperature, the slides then were rinsed with TBS containing 0·1% Tween-20. Streptavidin conjugated to alkaline phosphatase (Mabtech, Stockholm, Sweden) was applied for visualization of the biotinylated primary antibody for 30 min. Aminoethyl carbazole (Labvision, Fremont, CA) and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Moss Inc., Pasadena, MD), respectively, were used as substrates.

Manual double stains for CD45RA and CD3 and CD27 and CD3 were performed using immunofluorescence techniques. After deparaffinization and hydration, sections then were treated with 1 mM ethylenediaminetetraacetic acid (pH 8·0) and heated in a microwave oven for epitope retrieval as listed in Table 2. After cooling, sections were incubated for 20 min with standard blocking reagent (Roche). The primary monoclonal mouse anti-CD45RA (NeoMarkers) or anti-CD27 (NeoMarkers) antibodies were applied overnight at 4°. Sections were then rinsed in TBS buffer containing 0·1% Tween-20 (Merck) followed by application of goat anti-mouse indocarbocyanine (Cedarlane, Hornby, Ontario, Canada) for 30 min at room temperature. After rinsing, rabbit monoclonal anti-CD3 (NeoMarkers) antibodies were incubated overnight at 4°. Biotinylated swine anti-rabbit antibodies (Dako) were then incubated for 30 min. Then the sections were washed with TBS buffer containing 0·1%

Table 1. Characteristics of donors

	n	Male	Female	Age range (years)	Mean age ± SD (years)
Young	12	7	5	1–20	11 ± 5
Elderly	26	17	9	67–88	75 ± 5

Table 2. Antibodies and antigen retrieval techniques used

Antibody	Isotype	Dilution	Retrieval	Incubation	Source
CD3	Mouse monoclonal	1 : 50	citrate buffer pH 6, wet autoclave, 5 min, 121°	30 min, 20°	Dako
CD3	Rabbit monoclonal	1 : 150	1 mM EDTA pH 8, microwave oven, 10 min 950 W, 5 min 750 W	manual, overnight, 4°	NeoMarkers
CD4	Mouse monoclonal	1 : 10	citrate buffer pH 6, wet autoclave, 5 min, 121°	30 min, 20°	Novocastra
CD8	Mouse monoclonal	1 : 50	citrate buffer pH 6, wet autoclave, 5 min, 121°	30 min, 20°	Dako
CD20	Mouse monoclonal	1 : 700	citrate buffer pH 6, microwave oven, 10 min 800 W	30 min, 20°	Dako
CD20	Mouse monoclonal	1 : 50	citrate buffer pH 6, microwave oven, 10 min 950 W, 7·5 min 750 W, 7·5 min 650 W	manual, 60 min, 20°	NeoMarkers
CD27	Mouse monoclonal	1 : 200	1 mM EDTA pH 8, microwave oven, 10 min 950 W, 5 min 750 W	manual, overnight, 4°	NeoMarkers
CD45RA	Mouse monoclonal	1 : 500	–	30 min 20°	Dako
CD45RA	Mouse monoclonal	1 : 200	1 mM EDTA pH 8, microwave oven, 10 min 950 W, 5 min 750 W	manual, overnight, 4°	NeoMarkers
IgD	Rabbit polyclonal	1 : 150	citrate buffer pH 6, microwave oven, 10 min 950 W, 7·5 min 750 W, 7·5 min 650 W	manual, 60 min, 20°	Dako
IgM	Rabbit polyclonal	1 : 20 000	0·1% pronase, 8 min	30 min, 20°	Dako
IgG	Rabbit polyclonal	1 : 20 000	0·1% pronase, 4 min	30 min, 20°	Dako

Tween-20 and streptavidin–fluorescein isothiocyanate (Pharmingen) was applied. Stained slides were then analysed using confocal microscopy with a μ-Radiance confocal scanning system (Bio-Rad Laboratories, Hertfordshire, UK) attached to a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

T-cell clonality

The total DNA from the formalin-fixed paraffin-embedded tissue samples was extracted using a GenoVision (Qiagen, Hilden, Germany) extraction kit. For polymerase chain reaction analysis of the T-cell receptor (TCR) repertoire, the TCR-γ gene rearrangements were used as a marker of clonality.[17] Because of the restricted germ-line repertoire of the V gene segments, which limits the number of required primers, and as a result of the ordered hierarchy of TCR loci gene rearrangements this is a reliable tool for the detection not only of clonal TCR-γδ but also of TCR-αβ populations.[18] For this purpose we applied primers directed to Vγ10 and Vγ11 as well as Jγ11 and JP1 and the hot-start *Taq* DNA polymerase (Qiagen) in a thermal cycler (Eppendorf, Hamburg, Germany) in two separate parallel mixes for 45 cycles. The primers were synthesized by an automated DNA synthesizer (GenXpress, Maria Würth, Austria). DNA integrity was assessed by amplification of the Rhesus CE gene. The products were detected using the restriction fragment length polymorphism in a polynat gel matrix (Elchrom Scientific, Cham, Switzerland).

Image analysis and counting

The percentage of cells positive for CD4, CD8, CD20 and CD45 cells was determined by estimating the size of the area occupied by positive cells compared to a reference area of 4 mm^2 using a light microscope (Leica, Wetzlar, Germany). The number of IgM- and IgG-positive cells was counted in five random 0·747-mm^2 fields, and then extrapolated to a mean/mm^2. The planimetry of germinal centres and mantle zones was measured using the AXIOVISION imaging software (Carl Zeiss). The cumulative absolute size of three germinal centres and the corresponding mantle zones was assessed. The proportion of the size of the area occupied by germinal centre versus mantle zone was expressed as a percentage, the size of the whole area being considered as 100%. The percentage of $CD45RA^+$ $CD3^+$ and of $CD27^+$ $CD3^+$ cells was estimated at ×200 magnification using confocal microscopy and the AXIOVISION VIEWER.

Statistical analysis

Independent-sample *t*-test and analysis of variance (ANOVA), followed by post-hoc multiple comparisons, were used to compare group differences. To test the

association between variables, Pearson's linear regression analysis was performed. Analysis was carried out using the SPSS 10·0 software. *P*-values below 0·05 were considered statistically significant.

Results

Age-related changes in T- and B-cell subsets and antibody expression

We observed an age-related loss of the relative number of $CD8^+$ cells that was more dominant than the loss of $CD4^+$ cells (Fig. 1; Table 3). While regression analysis did not indicate a correlation between the proportion of lymph node $CD4^+$ T cells and age (data not shown), there was a significant negative correlation between the proportion of $CD8^+$ cells and age ($r = -0{\cdot}337$, $P = 0{\cdot}038$). The age-related changes in the proportions of $CD4^+$ and $CD8^+$ cells led to a significantly increased CD4/CD8 ratio in the lymph nodes of elderly people ($P < 0{\cdot}05$).

Comparing the single staining for CD45RA, there was a tendency towards decreasing numbers of positive cells in elderly individuals but this was not significant (Table 3).

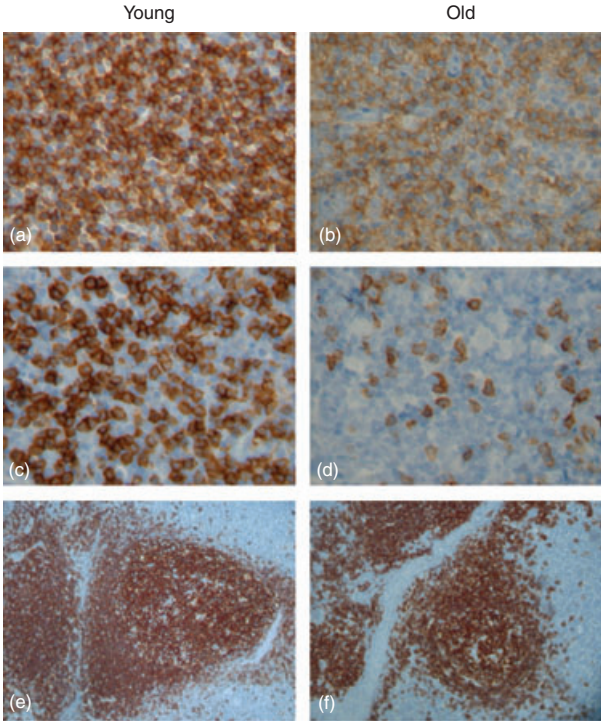

Figure 1. Immuperoxidase stainings of paraffin-embedded tissue. CD4-positive (a, b) and CD8-positive (c, d) cells in the paracortex area of lymph nodes from young (a, c) and old (b, d) donors; original magnification ×400. CD20-positive cells (e, f) which are predominantly located in follicles in young (e) and old (f) donors; original magnification ×40.

Table 3. Characteristic differences of variables between lymph nodes from young and elderly donors

	Young (*n*)	Elderly (*n*)	Significance
CD4 (%)	52·08 ± 13·73 (12)	46·54 ± 15·21 (26)	NS
CD8 (%)	26·25 ± 5·69 (12)	19·81 ± 8·77 (26)	$P < 0{\cdot}05$
CD20 (%)	23·75 ± 10·25 (12)	23·85 ± 10·79 (26)	NS
CD45RA (%)	45 ± 25·31 (12)	40·63 ± 17·59 (26)	NS
CD45RA/CD3	76 ± 11 (9)	10 ± 6 (7)	$P < 0{\cdot}01$
Ratio CD4/CD8	2·02 ± 0·6 (12)	2·68 ± 1·09 (26)	$P < 0{\cdot}01$
Relative number of follicles	36 ± 31 (12)	33 ± 17 (26)	NS
Diameter of follicles (μm)	260 ± 80 (12)	330 ± 240 (26)	NS
Proportion of germinal centre to mantle zone (%)	44 ± 13 (11)	32 ± 13 (24)	$P < 0{\cdot}05$
IgM (per mm^2)	29 ± 11 (4)	10 ± 9 (7)	$P < 0{\cdot}05$
IgG (per mm^2)	145 ± 124 (5)	110 ± 148 (8)	NS

Data presented as: mean ± standard deviation with number of samples in parenthesis. NS = not significant.

Immunofluorescence double staining for CD45RA and CD3 (Fig. 2) showed significantly increased proportions of $CD45RA^+$ $CD3^+$ coexpressing cells in younger individuals ($P < 0{\cdot}01$; Table 3). Regression analysis also demonstrated a strong negative correlation between $CD45RA^+$ $CD3^+$ coexpression and age ($r = -0{\cdot}961$, $P < 0{\cdot}01$). In addition, it was observed that $CD3^+$ cells almost exclusively coexpressed $CD27^+$. This demonstrates that $CD3^+$ $CD45RA^+$ T cells were naïve and not effector cells.

The size and number of follicles varied greatly between samples and we could not find age-related changes (Table 3). The relative proportion of germinal centre as compared to mantle zone did however, correlate negatively with age ($r = -0{\cdot}41$; $P = 0{\cdot}015$). Elderly individuals had smaller germinal centres than young people (Table 3). Although it did not reach statistical significance, the number of perifollicular B cells tended to be increased in lymph nodes of elderly people.

The number of IgM-expressing cells per mm^2 in the follicle also differed significantly between young and old individuals ($P = 0{\cdot}029$; Table 3). Young donors had higher numbers of IgM^+ cells, while the numbers of IgG^+ cells did not differ significantly between the age groups.

Effect of T-cell clonal expansions in the lymph nodes of young and elderly people

To analyse the effect of T-cell activation and consecutive clonal T-cell propagation on B cells in the lymph nodes from young and elderly people, we identified people with and without evidence of dominant T-cell clones in their lymph node tissue. We detected clonal T-cell expansions

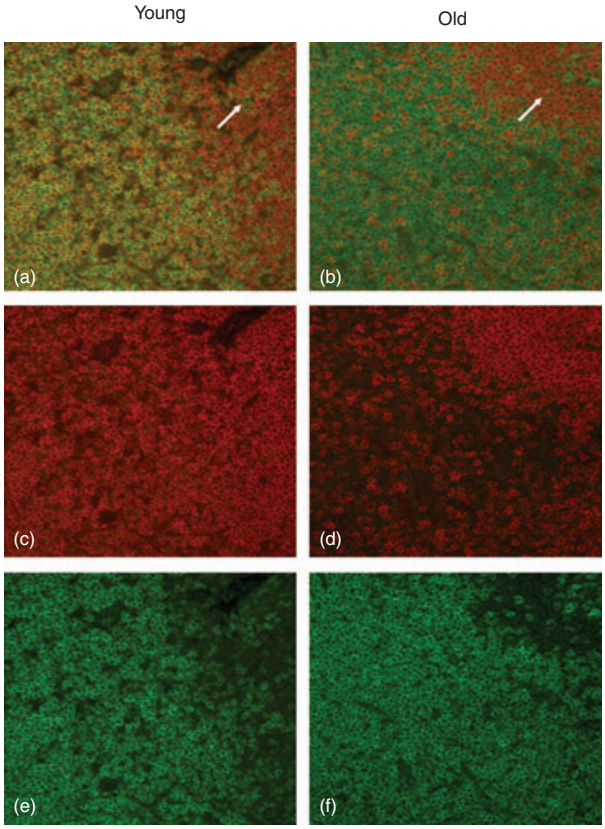

Figure 2. Immunofluorescence stainings of paraffin sections of lymph nodes. Stainings were performed with antibodies to CD3 (green) and CD45RA (red) and were analysed by confocal microscopy. Double-positive cells showed a mixed yellow colour. Lymph nodes from young people (a) contained more $CD45RA^+$ $CD3^+$ cells than lymph nodes from elderly people (b). A follicle which contains CD45RA (red) single-positive B cells is indicated by the white arrow. The red and the green channels of the same region are separately demonstrated in (c) to (f): (c, e) young donor, (d, f) old donor; original magnification ×200.

in the lymph nodes of 19% (5/26) of the elderly and 50% (6/12) of the young people in whom lymphoma had been excluded histologically and clinically (Fig. 3). We then compared the microarchitecture of the B-cell areas in tissue samples from people with and without clonal T-cell expansions. Young people with clonal T-cell populations had higher proportions of $CD20^+$ cells within their lymph nodes (Fig. 4a) and higher relative and absolute dimensions of their germinal centres compared to the mantle zone areas than young people without T-cell expansions in their lymph node tissue (Fig. 4b). Corresponding changes were not observed in the elderly group. In this group tissue samples with and without clonal T-cell expansions contained similar numbers of CD20-expressing cells and had a similar germinal centre to mantle zone relationship.

A significantly higher number of IgM-expressing cells was also observed in tissue samples from young donors with clonal T-cell expansions when compared to lymph

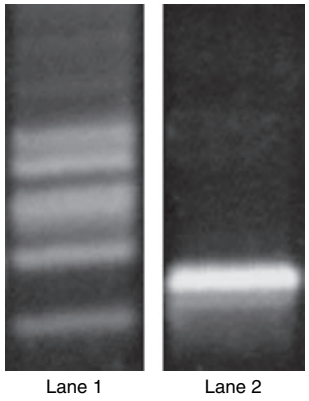

Figure 3. Restriction fragment length polymorphism analysis showing multiple smearing bands (lane 1) indicating a lack of dominant T-cell clones and a single band (lane 2) indicating the presence of a clonal T-cell population. Examples of lymph node tissue from one young person and from one elderly person are shown.

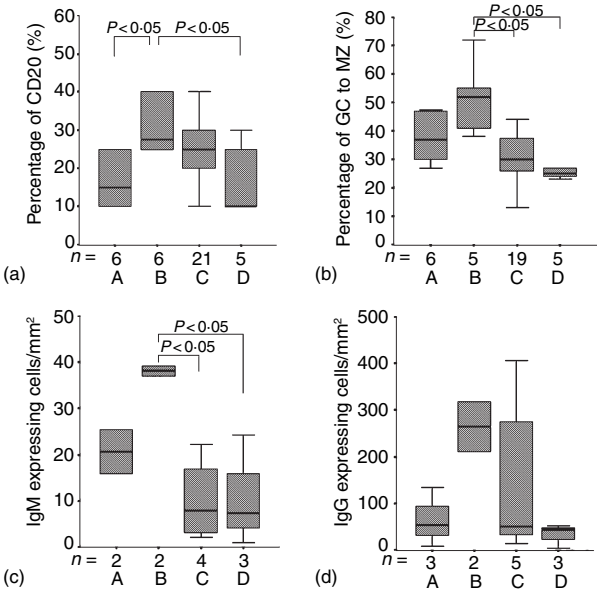

Figure 4. The box plots show the percentage of CD20-expressing cells (a), the proportion of the size of the germinal centre to the mantle zone area (b) as well as the numbers of IgM-expressing (c) and IgG-expressing (d) cells per mm^2 based on age groups as well as the presence or absence of clonal T-cell expansions: A–B, lymph nodes from young people, B–D, lymph nodes from elderly people; A and C without clonal expansion, B and D with clonal expansion.

node tissue from age-matched people without clonal expansions. In contrast, the number of IgM-producing cells was equally low in lymph nodes from elderly people with and without clonal T-cell populations (Fig. 4c). Samples from young people with clonal T-cell expansions also contained higher numbers of IgG-expressing cells. However, because there was only a small number of sam-

ples available for these stainings, the difference was not statistically significant (Fig. 4d).

Discussion

The declining function of the aging immune system has been the subject of many studies. However, most of them have been performed using human peripheral blood samples or animal models. Here, we present a morphometrical, immunohistochemical, immunofluorescent and molecular analysis of the age-related changes in the T- and B-cell repertoire in human lymph nodes and demonstrate age-related alterations.

In the T-cell repertoire we found fewer $CD8^+$ and $CD45RA^+$ naïve T cells in the lymph nodes with increasing age. A decrease of naïve T-cell numbers related to age has been well documented in the peripheral blood[19] and is believed to be the result of thymic involution in combination with ongoing differentiation of naïve T cells into antigen-experienced memory or effector cells. This seems to lead to a 'switch' towards memory/effector $CD45RO^+$ T cells which may eventually leave the lymph node to migrate to peripheral non-lymphoid organs.[19,20] The reduction in the size of the naïve T-cell repertoire may have an impact on the responsiveness of the immune system to new antigens in the elderly.

Our data also showed an age-dependent increase in the CD4/CD8 ratio. Some previous studies reported an increased CD4/CD8 ratio in the peripheral blood of patients with multiple sclerosis and Alzheimer's disease.[21,22] A decreased CD4/CD8 ratio in the lymph nodes of aging mice has also been reported.[23]

Banerjee and co-authors found that in the human spleen and gut there was no apparent correlation between the size of germinal centres, mantle zones, or marginal zones and age; they noted a decreased proportion of $CD8^+$ cells in the spleen T-cell zone, but not in gut-associated lymphoid tissue.[24] Preferential loss of $CD8^+$ cells in secondary lymphoid tissues with age could be because the production of $CD8^+$ cells is more affected by thymic involution than that of $CD4^+$ cells.[24,25] A similar constellation has been observed in the peripheral blood of aged individuals.[26]

Our data revealed no difference in the number and size of follicles or the percentage of $CD20^+$ B cells between young and old individuals. Similarly, in mice it has been suggested that the total number of B cells does not decrease with age, but that the B cells show an impaired responsiveness to foreign antigens, though the number of immunoglobulin-secreting cells and the level of serum immunoglobulin does not decline.[27,28] A study in mice showed that the number and volume of germinal centres decline with increasing age. These declines could have implications for the reduction of the germinal centre reaction with age.[14]

When young individuals with clonal T-cell populations in their lymph node tissue were analysed, we observed an increased number of $CD20^+$ B cells within the lymph nodes, an increase in the size of germinal centres as well as an increased number of IgM-expressing cells. Taking into consideration the fact that lymph node dissection was performed in young individuals because of lymphadenopathy and that lymphoma and other specific causes for lymph node enlargement were clinically and histologically excluded, infection appeared to be the most probable cause of lymph node swelling. We speculate that in response to the infectious stimulus in these individuals, a B-cell reaction with prominent germinal centres and shrinking mantle zones and IgM production as well as clonal T-cell expansion occurred. As already known, the germinal centre is the critical microenvironment for B-cell activation and differentiation in response to antigenic stimulation.[29] Thus, our observation probably represents a morphological correlate of the physiological immune response. In contrast, old donors with clonal T-cell populations had relatively low percentages of $CD20^+$ B cells and a 'static' follicular microarchitecture, suggesting that these clonal T-cell populations are probably not functional in a way that they are able to induce histologically detectable changes in the B-cell compartment or may even represent 'dormant' T-cell lymphomas. Although the exact mechanisms responsible for the observed age-associated defect in T-cell/B-cell interactions is not yet known, one has to assume that expanded T-cell clones obviously fail to provide sufficient activation signals to adjacent B cells in elderly people.

Taken together, our findings of decreased naïve T-cell numbers in the lymph nodes and of probable alterations in T-cell/B-cell interactions may provide further insight into the mechanism of immune dysfunction in the elderly.

Acknowledgements

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